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Aqueous fraction from *Costus spiralis* (Jacq.) Roscoe leaf reduces contractility by impairing the calcium inward current in the mammalian myocardium

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ABSTRACT

Ethnopharmacological relevance: Brazilian folk medicine uses infusion of *Costus spiralis* leaf to help people to treat arterial hypertension and syndromes of cardiac hyperexcitability.
Aim of the study: Evaluate the aqueous fraction (AqF) effect on atrial contractility and investigate its mechanism of action.
Materials and methods: The AqF effect on the cardiac contractility was studied on isolated electrically driven guinea pig left atria. Atropine and tetraethylammonium (TEA) were employed to investigate whether potassium contributes for the inotropic mechanism of the AqF. The role of calcium in this effect was also studied. This was done by analysing the AqF effect on the Bowditch's phenomenon, as well as by studying whether it could interfere with the concentration–effect curve for $CaCl_2$, isoproterenol, and BAY K8644. Mice isolated cardiomyocytes were submitted to a whole-cell patch-clamp technique in order to evaluate whether the L-type calcium current participates on the AqF effect. Furthermore, the intracellular calcium transient was studied by confocal fluorescence microscopy.
Results: AqF depressed the atrial contractile force. It was the most potent fraction from *C. spiralis* leaf (EC_{50} = 305 ± 41 mg/l) (crude extract: EC_{50} = 712 ± 41; ethyl acetate: EC_{50} = 788 ± 121; chloroform: EC_{50} = 8948 ± 1346 mg/l). Sodium and potassium content in the AqF was 0.15 mM and 1.91 mM, respectively. Phytochemical analysis revealed phenols, tannins, flavones, xanthones, flavonoids, flavonols, flavononols, flavonones, and saponins. Experiments with atropine and TEA showed that potassium does not participate of the inotropic mechanism of AqF. However, this fraction decreased the force overshoot characteristic of the Bowditch's phenomenon, and shifted the concentration–response curve for $CaCl_2$ (EC_{50} from 1.12 ± 0.07 to 7.23 ± 0.47 mM) indicating that calcium currents participate on its mechanism of action. Results obtained with isoproterenol (1–1000 pM) and BAY K8644 (5–2000 nM) showed that AqF abolished the inotropic effect of these substances. On cardiomyocytes, 48 mg/l AqF reduced (~23%) the L-type calcium current density from −6.3 ± 0.3 to −4.9 ± 0.2 A/F (n = 5 cells, $p < 0.05$) and reduced the intracellular calcium transient (~20%, 4.7 ± 1.2 a.u., n = 42 cells to 3.7 ± 1.00 a.u., n = 35 cells, $p < 0.05$). However, the decay time of the fluorescence was not changed (control: 860 ± 32 ms, n = 42 cells; AqF: 876 ± 26 ms, n = 35 cells, $p > 0.05$).
Conclusions: The AqF of *C. spiralis* leaf depresses myocardial contractility by reducing the L-type calcium current and by decreasing the intracellular calcium transient. Despite the lack of data on the therapeutic dose of AqF used in folk medicine, our results support, at least in part, the traditional use of this plant to treat cardiac disorders.

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1. Introduction

Costus spiralis (Jacq.) Roscoe, a member of the Zingiberaceae family, is commonly found in South American countries. In the Brazilian Amazon, the popular name for *C. spiralis* is Cana-do-Brejo. Phytochemical analysis has revealed the presence of saponins (Da Silva and Parente, 2004) and flavonoids (Antunes et al., 2000). Furthermore, the presence of inulin, oxalic acid, tannins, systosterol, sapogenins, mucilage, and pectin has been noted by Albuquerque (1989), Corrêa et al. (1998), and Vieira and Albuquerque (1998). In the methanolic fraction Braga et al. (2007) reported the following constituents: flavonoids, esteroids, and alkaloids. *C. spiralis* has been used as an ornamental plant and employed in folk medicine (Di Stasi and Hiruma-Lima, 2002; Araújo and Oliveira, 2007) to treat hypertension and renal dysfunction and as a diuretic agent (Cruz, 1982; Di Stasi and Hiruma-Lima, 2002). The antiurolithiatic activity of this plant was confirmed by Viel et al. (1999), corroborating its traditional use. Furthermore, its use as an antimicrobial, antifungic, and antioxidant (Habsah et al., 2000; Braga et al., 2007) agent, as well as its antileishmanial, cytotoxic, antiinflammatory, and immunomodulatory activity (Whittle, 1964; Silvia et al., 2000; Silvia and Parente, 2003) has been reported in the literature. Pio-Corrêa (1984) also described the utility of *C. spiralis* juice in the treatment of tachycardia. Kistler and Obeyesekere (2007) observed that this kind of arrhythmia increases the likelihood of myocardial infarction, heart failure, and cardiac strokes. Generically, tachycardias are classified as ventricular or supraventricular, both of which have tremendous clinical relevance.

Supraventricular tachycardias, such as those due to atrial fibrillation are commonly seen in medical practice. In most instances, atrial reentry mechanisms, often involving the atrioventricular node, are essential for sustaining the abnormal rhythm (Lee et al., 2008; Medi et al., 2009). Although a relatively large number of drugs, such as the Ca^{2+}-channel antagonists, are used to treat supraventricular tachycardias, the pharmacological management of this condition is still very complicated, mainly due to undesirable drug side effects. Because *C. spiralis* is a plant traditionally used to treat tachycardias, we decided to determine if its use in folk medicine could be scientifically supported. Therefore, we began by investigating whether fractions obtained from a crude extract of the *C. spiralis* leaf can alter mammalian atrial contractility. Furthermore, our work provided new insights into the functional and cellular mechanisms of the aqueous fraction in heart muscle.

2. Materials and methods

2.1. Fractions

Leaves from *C. spiralis* were collected from a healthy and agrotoxic-free plant (10°56′42.14″S, 37°03′09.17″O, Aracaju, State of Sergipe, Brazil) during the winter season (June, 2009). Botanical identification was performed by Dr. Maria Olivia de Oliveira Cano of the Herbarium of Instituto Agronômico de Pernambuco "Dárdano de Andrade Lima", where a voucher specimen was deposited (#70.285). Leaves were gently cleaned, washed, and dried (48 ± 2 °C, 8 days) before being subjected to maceration in a water:ethanol solution (1:1, v:v, 27 ± 2 °C, for 15 days). The solvents were evaporated in a Soxhlet apparatus (Laborota 4000, Heidolph, UK) and the hydroethanolic extract, designated as crude extract (CEx), was stored at −21 °C. From 222.30 g of dry leaves, we obtained 23.40 g of CEx (10.50%). Then 7.65 g of CEx were subjected to liquid–liquid partition employing three solvents, chloroform (ChloF), ethyl acetate (EtAF), and water (AqF), to give the following yields: 130.08 mg (1.70%), 469.41 mg (6.14%), and 5309.13 mg (69.40%), respectively. The solvents were evaporated (50 ± 2 °C), and all fractions were stored at −21 °C prior to use.

2.2. Chemical analysis of C. spiralis aqueous fraction

Sodium and potassium content of the AqF was determined by flame photometry (Digimed DM 61, São Paulo, SP, Brazil). The main classes of secondary metabolites were determined using the Matos protocol (1997).

2.3. Animals

Animals were handled according to procedures set forth by the National Council of Control of Animal Experimentation (CONCEA/MCT, Brazil). The animal protocol was previously approved (Protocol #038/2008) by the Ethical Committee on Animal Research of the Federal University of Sergipe. Guinea pigs of both sexes (300–500 g) were supplied by the animal care unit of the Federal University of Sergipe. Intracellular calcium transients, as well as measurements of the calcium inward current, were performed on isolated cardiomyocytes from male C57BL6/J mice weighting approximately 30 g. The experiments were approved by the Ethical Committee on Research of the Federal University of Minas Gerais (Protocol #236/2009).

2.4. Drugs

Reserpine, (−) BAY K8644 (1,4-dihydro-2-6-dimethyl-5-nitro-4-[2-(trifluoromethyl)-phenyl]-3-pyridine acid carboxylic methyl ester), isoproterenol, atropine, tetraethylammonium chloride (TEA-Cl), EGTA, HEPES, cesium chloride (CsCl), barium chloride ($BaCl_2$), cesium hydroxide (CsOH), protease type XXIII, porcine trypsin, lactic acid, pyruvic acid, and DMEM ("Dulbecco's Modified Eagle's Medium") were purchased from Sigma-Aldrich (St. Louis, MO, USA), and collagenase type II was purchased from Worthington Biochemical Corporation (Freehold, NJ, USA). Heparin was purchased from ROCHE (Rio de Janeiro, RJ, Brazil), and Fluo-4 AM was purchased from Molecular Probes (Eugene, OR, USA).

2.5. Atrial contractility

Guinea pig atria were isolated, mounted in an organ chamber (5 ml, 29 ± 0.1 °C), and superfused with modified Tyrode's solution (pH 7.12) gassed with carbogen (Dorigo et al., 1990; Vasconcelos et al., 2005). The inotropic effects of CEx and its subfractions were determined by adding them cumulatively to the organ bath. The atria were electrically driven by field stimulation employing suprathreshold pulses of 0.5 ms.

2.6. Evaluation of the effect of AqF on calcium entry in myocardial cells

2.6.1. Evaluation using the Bowditch maneuver

The positive staircase phenomenon described by Bowditch (1871) is characterized by an increase in the amplitude of myocardial contractile force following a sudden increase in the stimulation rate. This is due to an increase in calcium entry into myocardial cells, as demonstrated by Nayler and Merrillees (1971). To implement the experimental protocol used by these authors, guinea pigs were injected with reserpine (5 mg/kg, via i.p.) 24 h prior to the experiments. Briefly, the protocol was as follows: (1) the atrium was stimulated at a very low rate (0.2 Hz, control stimulation) until contractile force reached a steady-state; (2) then suprathreshold stimuli were abruptly applied to overdrive the myocardial tissue at various test rates; this procedure unbalanced the atrium and caused the myocardial tissue to develop additional contractile force. The test rate was maintained for 150 s. Then stimulation was paused for 40 s before returning to the control rate. The applied test rates

were (Hz) 0.33, 0.50, 0.66, 1.00, 1.33, 1.66, 2.00, 2.50, and 3.33. To study the effect of the most potent fraction on calcium entry into myocardial cells, the Nayler and Merrillees protocol was performed before and after exposing the atrium to the aqueous fraction of *C. spiralis*.

2.6.2. Evaluation using $CaCl_2$, isoproterenol, and (−) BAY K8644

To study the effect of the most potent fraction of *C. spiralis* on myocardial contractility, experimental maneuvers were performed to increase the calcium inward current. This was achieved by exposing the atrium to different calcium concentrations, or by adding isoproterenol or (−) BAY K8644 to the organ bath. In each case, concentration–response curves were obtained before and after incubating the atrium with the most potent fraction of *C. spiralis*.

2.7. Cardiomyocyte isolation

Ventricular cardiomyocytes from mice (C57BL6/J) were enzymatically isolated as previously described by Shioya (2007).

2.8. L-type calcium current measurements

Whole-cell voltage-clamp recordings were obtained at room temperature (22–25 °C) using an EPC-9.2 patch clamp amplifier (HEKA Electronics, Rheinland-Pfalz, Germany) as has been previously described (Lauton-Santos et al., 2007; Oliveira et al., 2007; Roman-Campos et al., 2009; Lara et al., 2010). For $I_{Ca,L}$ measurements, recording pipettes were filled with internal solution containing (in mM) 5 NaCl, 120 CsCl, 20 TEA-Cl, 5 EGTA, and 10 HEPES (pH set to 7.2 with CsOH). $I_{Ca,L}$ was recorded in the presence of 1.8 mM extracellular Ca^{2+} (external solution).

For the time-course analysis of the effect of the most potent *C. spiralis* fraction on the L-type Ca^{2+} channels, $I_{Ca,L}$ was elicited every 10 s by test pulses generated from a holding potential of −80 mV to −40 mV for 50 ms to inactivate the Na^+ and T-type Ca^{2+} channels. Then we stepped up the membrane potential to 0 mV for 200 ms. This protocol was repeated before, during, and after washing out the most potent fraction. Data are presented as current density ($I_{Ca,L}/C$, where C is the cell capacitance).

2.9. Sarcoplasmic calcium removal

Myoplasmic calcium removal occurring after development of the peak twitch contractile force was evaluated using the Conde-Garcia equation, $F(i) = ((100 - F_{min})/K^i) + F_{min}$, where $F(i)$ is the amplitude of the ith contraction after a pause; F_{min} is the minimal amplitude of the post-pause contractions; and K is the decay time-constant for the post-pause contractions. This equation was employed to fit the atrial twitch contractions obtained after the pause in stimulation (Nayler and Merrillees, 1971). The Conde-Garcia equation when expressed as $\log(F(i) - F_{min}) = \log(100 - F_{min}) - i \log(K)$ results in a straight line described by an equation of the type $Y = B - Ax$, where $Y = \log(F(i) - F_{min})$, $B = \log(100 - F_{min})$, $A = \log K$, and $X = i$. A is the slope of the straight line and represents the decay rate during the post-pause phase. We interpret this parameter as a descriptor of the calcium removal rate from the sarcoplasm, a phenomenon that is associated with the myocardial relaxation phase.

2.10. Intracellular calcium transient

Isolated cardiomyocytes obtained from mouse ventricles were loaded with 5 μM Fluo-4 AM (stock solution prepared in pure dimethyl-sulfoxide – DMSO) for 30 minutes at room temperature and then washed with extracellular solution containing 1.8 mM Ca^{2+} to remove the excess of dye (Lauton-Santos et al., 2007). Cardiac cells were exposed to the most potent *C. spiralis* fraction for 3 min. Then Ca^{2+} transients were elicited by electrical field stimulation applied to the cardiomyocytes through a pair of platinum electrodes. Suprathreshold square pulses of 0.2 ms duration were continuously applied at 1 Hz. A Meta LSM 510 scanning system (Zeiss GmbH, Jena, Germany) with a 63× oil immersion objective and 10× ocular was used for confocal fluorescence imaging with a total magnification of 630×. Fluo-4 AM was excited at 488 nm (Argon laser), and the emission intensity was measured at 510 nm. For recording Ca^{2+} transients, myocytes were scanned with a 512 pixel line. The scan line was positioned randomly along the longitudinal axis of the cell, although care was taken to avoid crossing the nuclei. Cells were scanned every 1.54 ms, and sequential scans were stacked to create two-dimensional images with time on the x-axis. Digital image processing was performed using a custom-designed routine in the IDL programming language (Research Systems, Boulder, CO). The intracellular Ca^{2+} levels were reported as F/F_0, where F_0 is the resting Ca^{2+} fluorescence.

2.11. Statistical analysis

Data are reported as the mean ± S.E.M. Figures were created in GraphPad Prism (GraphPad Software, CA, USA), Excel (Microsoft Office, USA), or SigmaPlot (Systat Software, IL, USA). Mean values were compared using the paired Student's t-test or one-way ANOVA. $p < 0.05$ was used as the criterion for statistical significance.

3. Results

3.1. Effect of C. spiralis fractions on atrial contractility

CEx and the other *C. spiralis* fractions depressed atrial contractility in a concentration-dependent manner. However, the potencies of the various fractions, as estimated by EC_{50}, were not equal. Fig. 1(A–D) shows representative recordings of the effect produced by *C. spiralis* fractions on atrial contractile force. Fig. 1E summarizes those effects. The following EC_{50} values were found: 305 ± 41 mg/l for AqF, 712 ± 41 mg/l for CEx, 788 ± 121 mg/l for EtAF, and 8948 ± 1346 mg/l for ChloF. Although all *C. spiralis* fractions produced a negative inotropic effect, the AqF was the most potent. For this reason, it was used in the experiments subsequently carried out to evaluate the inotropic mechanism of *C. spiralis* in heart muscle.

We also investigated whether muscarinic receptors participate in the negative inotropic effect of *C. spiralis* AqF. Experiments were performed using atrial tissue pre-incubated with atropine (1.5 μM), a non-selective antagonist of muscarinic receptors. The presence of atropine did not alter the pharmacological effects of AqF (data not shown). The participation of membrane potassium channels in the cardiodepressor effect of *C. spiralis* was also investigated using TEA (20 mM), a nonspecific potassium channel blocker. In the presence of TEA, the negative inotropic effect of AqF was not modified. Thus, we may conclude that *C. spiralis* reduces cardiac contractility through a mechanism that does not involve potassium channels or the activation of cardiac muscarinic receptors.

3.2. Ionic and phytochemical content

The sodium and potassium content in 1 g/l of AqF, as determined by flame spectrometry, were 0.15 and 1.91 mM, respectively. These concentrations were not sufficient to produce an inotropic effect, even at the highest AqF concentration used in this study. The main classes of secondary metabolites present in AqF were phenols,

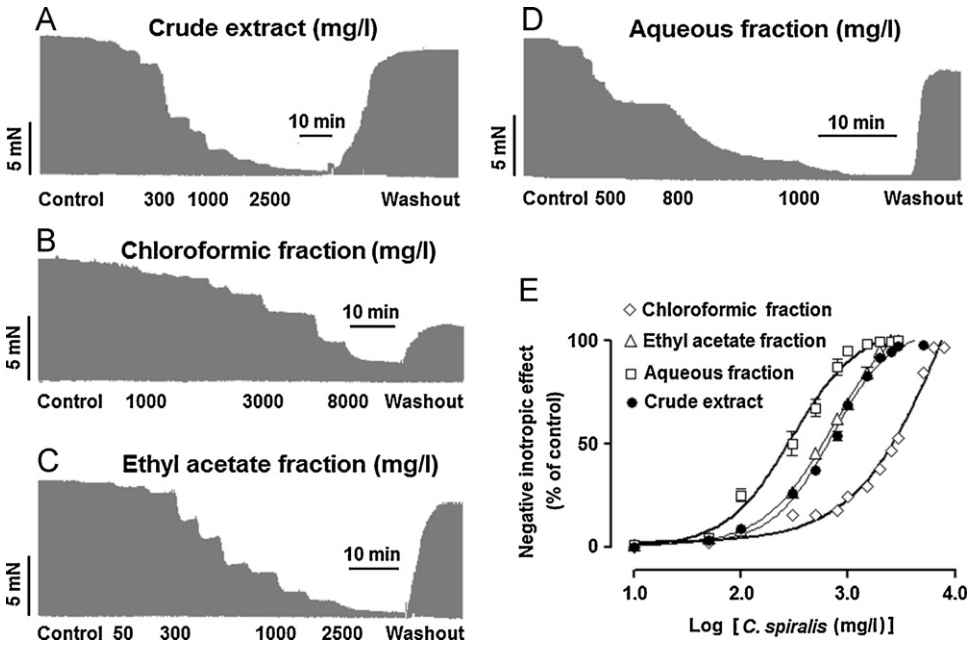

Fig. 1. Representative recordings showing the effect of *C. spiralis* leaf fractions on atrial contractile force (A, B, C, D) and concentration–response curves comparing the negative inotropic potency of the various *C. spiralis* leaf fractions (E). The experimental points were fitted by the Hill–Langmuir equation. AqF was the most potent fraction (EC_{50} = 305 ± 41 mg/l), and ChloF was the least potent (EC_{50} = 8948 ± 1346 mg/l) (27 ± 0.1 °C; field stimulation, 2 Hz, suprathreshold pulses, 0.5 ms; n = 3).

tannins, flavones, xanthones, flavonoids, flavonols, flavononols, flavonones, and saponins.

3.3. Effects of the aqueous fraction of *C. spiralis* on sarcolemmal calcium influx

The amount of calcium entering cardiomyocytes via the sarcolemma is a very important factor contributing to control of myocardial contractile force (Bers, 2008). To determine whether AqF modifies calcium influx, the experiments described in the next subsections were performed.

3.3.1. Bowditch phenomenon

The positive staircase phenomenon previously described by Bowditch (1871) is characterized by an increase in the amplitude of myocardial twitch contractions following a sudden increase in the stimulation rate. It was later demonstrated by several authors that this increase in contractile force is due to an increase in calcium entry into myocardial cells (Nayler and Merrillees, 1971; Endoh, 2004; Monasky and Jansen, 2009). In Fig. 2A, the additional force (i.e., the force overshoot) is plotted against the rate of stimulation. We can see that when the stimulation rate was gradually increased from 20 to 120 bpm, the overshoot of force increased continuously. Although this occurred in the control and washout conditions, the force overshoot was abolished when the atrium was incubated with 1500 mg/l AqF. This result suggests that AqF from *C. spiralis* acts by reducing calcium influx in cardiac muscle.

3.3.2. Evaluation by varying the external calcium concentration

Several experimental protocols were performed to test the effect of AqF on the calcium inward current. For us, this is the primary event supporting evidence for the negative inotropic effect of AqF in the myocardium. First, we followed a very straightforward protocol in which the extracellular calcium concentration was changed in the absence and in the presence of 1500 mg/l AqF.

Fig. 2C shows the positive inotropic effect of increases in extracellular calcium concentration. The EC_{50} for $CaCl_2$ was 1.12 ± 0.07 mM. However, when the atria were pre-incubated with 1500 mg/l AqF, the concentration–response curve shifted to the right, and the EC_{50} increased to 7.23 ± 0.47 mM (approximately 6 fold, $p < 0.05$). These data confirm that the cardiac effects of *C. spiralis* AqF are associated, at least in part, with a decrease in sarcolemmal calcium influx.

3.3.3. Evaluation using isoproterenol or (−) BAY K8644

It is well established that calcium influx in cardiac cells occurs by activation of both L-type (LCC) and T-type calcium channels (TCC) (Bers, 2008). To determine whether LCCs are involved in the cardiodepressor effect of AqF, we performed experiments using standard drugs to evoke positive inotropic responses by directly [(−) BAY K8644] or indirectly (isoproterenol) activating LCC. Cumulative addition of these drugs to the organ bath caused a positive inotropic effect on the myocardium (Fig. 2B and D). The EC_{50} calculated for isoproterenol and (−) BAY K8644 was 11.2 ± 1.2 pM and 77.2 ± 1.1 nM ($p < 0.05$), respectively. The positive inotropic effect of both isoproterenol and (−) BAY K8644 was completely abolished by the addition of 1500 mg/l AqF to the bath. Our findings suggest that the *C. spiralis* AqF reduces the L-type calcium is reduced by the *C. spiralis* AqF.

3.3.4. Evaluation using direct measurement of L-type calcium current

Further evidence indicating that AqF reduces the L-type calcium current in myocardial cells was obtained using the patch-clamp technique. Fig. 3A shows the time course of the effect of 48 mg/l AqF on L-type calcium current density in ventricular myocytes evoked at a rate of 0.1 Hz by depolarizing pulses to 0 mV from a holding potential of −80 mV. Our results showed that AqF reduced (by ~23%) the current density from −6.6 to −5.1 A/F, and the effect of AqF was partially reversible. Fig. 3B shows representative traces of the L-type calcium current obtained at the indicated time points in Fig. 3A. The recordings shown were obtained under control conditions (black trace), in 48 mg/l AqF (blue trace), and during washout (red trace). Fig. 3C summarizes the effect of AqF (48 mg/l) on the

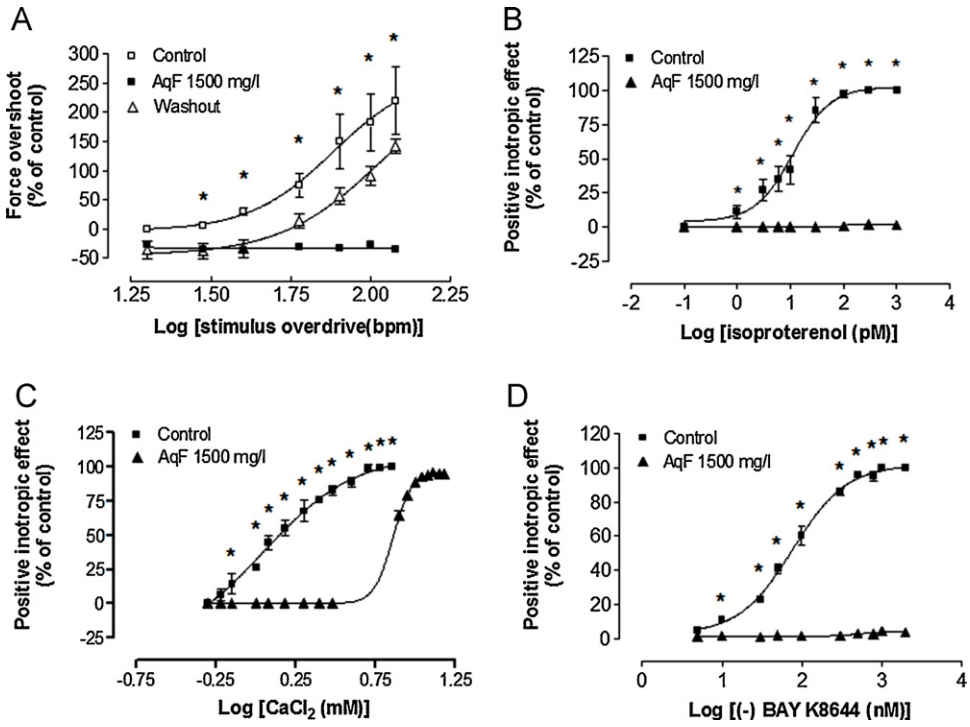

Fig. 2. AqF of *C. spiralis* decreases calcium entry in myocardial atrial preparations. This fraction completely abolished the Bowditch phenomenon (A), and the positive inotropic effect of both isoproterenol (B) and BAY K8644 (D). AqF also caused a rightward shift in the concentration–response curve for $CaCl_2$ (C) ($n = 4$; $^*p < 0.05$; $29 \pm 0.1\,^\circ C$).

L-type calcium current density; this fraction significantly reduced the L-type calcium current density from -6.3 ± 0.3 to -4.9 ± 0.2 A/F ($n = 5$, $p < 0.05$). Taken together, our results support the hypothesis that the reduction in cardiac contractile force promoted by the AqF is indeed a consequence of its inhibitory effect on L-type calcium channels.

3.4. Effects of the aqueous fraction of *C. spiralis* on the global intracellular calcium transient

It is well established that intracellular [Ca^{2+}] handling is critical for controlling cardiac contractility (Bers, 2008) and is also linked to arrhythmogenic mechanisms in the heart (Wang and Goldhaber,

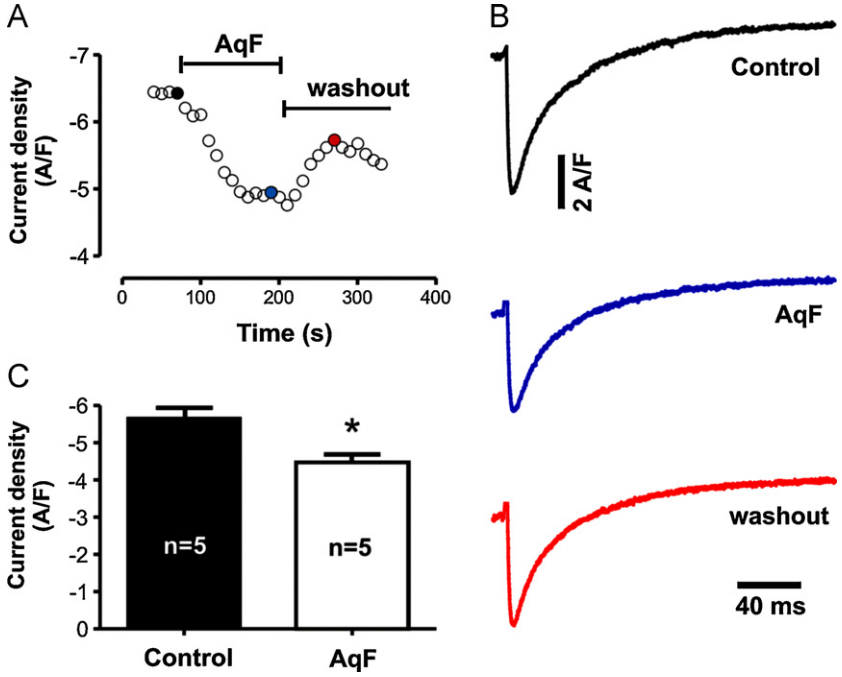

Fig. 3. *C. spiralis* AqF reduces L-type calcium current density in isolated mouse cardiomyocytes. *Panel A*: Time course of the calcium current (expressed as current density) in control solution (black circle), in AqF (blue circle), and after washout (red circle). *Panel B*: calcium current traces obtained under control conditions, in the presence of 48 mg/l AqF, and after washout. *Panel C*: L-type calcium current density measured at 0 mV under control conditions and in the presence of 48 mg/l AqF ($n = 5$ cells; 5 animals; $^*p < 0.05$; $29 \pm 2\,^\circ C$).

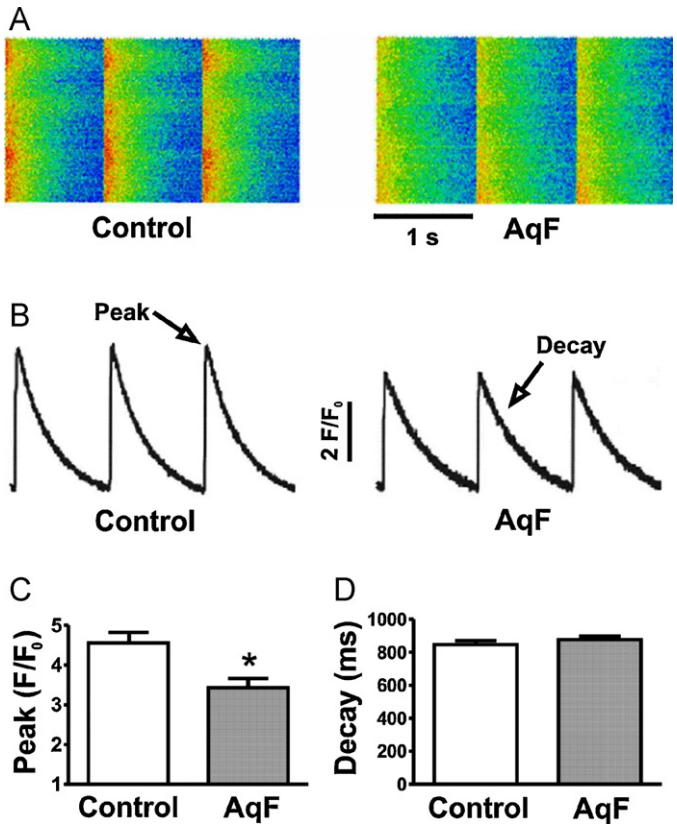

Fig. 4. Effect of AqF (48 mg/l) on the peak amplitude of the intracellular calcium transient and on the fluorescence decay time. Representative recordings of the intracellular calcium transient are seen in panels A and B. Panel A: pseudocolor representation of the intracellular calcium levels in the control and after 48 mg/l AqF. Panel B: reduction in the peak fluorescence after addition of 48 mg/l AqF to the external medium. Panels C and D: effect of AqF on the peak fluorescence signal and on the decay phase (at 90%) obtained in a group of cells (control, $n = 42$ cells; AqF, $n = 35$ cells; 4 animals; $^{*}p < 0.05$; field stimulation, suprathreshold pulses, 1 Hz).

2004). Because AqF induces a negative inotropic effect by inhibiting the L-type calcium current, we decided to investigate whether the global intracellular calcium transient was also affected by that fraction. Fig. 4A shows on a pseudocolor scale, the temporal changes in the intracellular calcium concentration observed in electrically stimulated cardiomyocytes. Data were obtained before (control) and after incubation of the cell with 48 mg/l AqF. The pseudocolor scale shows the range of the intracellular calcium concentration from low (blue) to high (red). The fluorescence was expressed as F/F_0, where F_0 is the fluorescence during the resting state and F is the maximal fluorescence measured after the delivery of the stimulus. As can be seen, the red color distribution was diminished in the presence of AqF, indicating a reduction in intracellular calcium levels. Fig. 4B depicts the original recordings of the intracellular calcium transient. In these traces, the peaks (arrow in the left panel) represent the highest level of intracellular calcium, whereas the decay phase (arrow in right panel) is associated with calcium removal from the sarcoplasm. This event reflects the activity of the Ca-ATPase of the sarcoplasmic reticulum (SERCA). Fig. 4C shows that AqF significantly changed the peak amplitude of the intracellular calcium transient, reducing it from 4.7 ± 1.2 (control, $n = 42$) to 3.7 ± 1.00 a.u. (AqF, $n = 35$, $p < 0.05$), a decrease of ~20%. In spite of that effect, the decay phase was unchanged, indicating that AqF did not alter the activity of the SERCA pump (Fig. 4D). The average time between peak fluorescence and 90% decay (T90) was 860 ± 32 ms ($n = 42$) in control and 876 ± 26 ms in the presence of AqF ($n = 35$, $p > 0.05$).

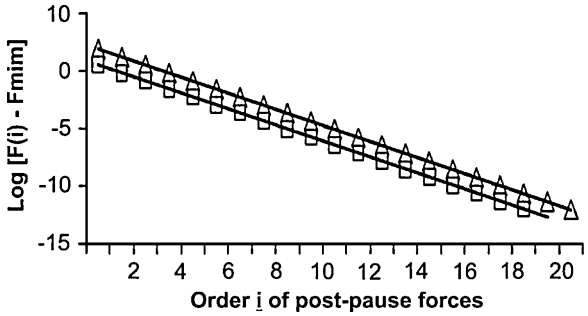

Fig. 5. Decay rate of the envelope of post-pause contractions, as measured with the aid of the Conde-Garcia equation. Angular coefficients were the same for the straight lines obtained in the control (△) and after adding AqF to the bath (□) ($n = 4$; $p > 0.05$; 29.0 ± 0.1 °C; Field stimulation: suprathreshold pulses, 0.5 ms, 0.2 Hz).

If the AqF does not affect SERCA activity, as suggested by the intracellular fluorescence signal, then the decay rate of the envelope of post-pause twitch contractions, as seen with the Nayler and Merrillees protocol, should not be affected by AqF. To evaluate this hypothesis, we analyzed the results obtained with that protocol using the Conde-Garcia equation. Fig. 5 depicts the results obtained before and after atrial incubation with AqF. Because the slope (angular coefficient) of both straight lines are the same, it is reasonable to conclude that these results corroborate those obtained with intracellular fluorescence.

In conclusion, the reduction in L-type calcium current was accompanied by a decrease in the global intracellular calcium transient without significant alterations in the kinetics of sarcoplasmic calcium removal. Therefore, our findings provide strong evidence to explain how the cardiodepressor effect of *C. spiralis* AqF occurs.

4. Discussion

In the present study, we have demonstrated that fractions obtained from the *C. spiralis* leaf promote a concentration-dependent cardiodepressor effect on the mammalian myocardium. The first step taken to better understand their contractile effect was to determine the most potent fraction. To do this, we compared the inotropic effects of the crude hydroethanol extract with those of the following fractions: ethyl acetate, chloroform, and water. All fractions depressed atrial contractility in a concentration-dependent fashion. However, the AqF exhibited the greatest potency. This suggests that the cardioactive compound(s) present in the AqF must be highly polar. The negative inotropic effect of the *C. spiralis* fractions and the comparatively high potency of AqF accord with the traditional use of this medicinal plant, wherein infusions and juices from its leaves are known to be prepared in water to treat patients suffering cardiac hyperexcitability syndromes.

Phytochemical screening performed on the AqF revealed the presence of the following classes of secondary metabolites: flavonoids, xanthones, and tannins. Its sodium and potassium content, however, was very low. At the highest concentration employed in this study, the AqF changed the extracellular sodium concentration from 120 to 120.15 mM and the potassium concentration from 1.91 to 4.61 mM. These maximal concentrations were insufficient to modify any of the atrial contractility parameters, as previously shown by our group (Vasconcelos et al., 2005).

It is well known that the activation of muscarinic M_2 receptors in atrial muscle causes an increase in the acetylcholine-sensitive potassium current leading to a reduction in action potential duration and membrane hyperpolarization. In addition, stimulation of M_2 receptors also inhibits membrane adenylate cyclase, which causes augmentation of the intracellular cyclic AMP level. These cellular effects contribute to the classical inotropic negative

response evoked by muscarinic agonists, principally through the reduction of calcium inward current (Dhein et al., 2001). Based on our results, we can rule out the possibility of muscarinic receptors and/or potassium channels mediating the cardiodepressor effect of *C. spiralis* AqF because incubation of the preparations with atropine, a non-selective muscarinic antagonist, or TEA, a nonspecific potassium channel blocker, did not modify the myocardial effects of *C. spiralis* fractions.

C. spiralis is a plant traditionally used to treat hypertension and disturbances in cardiac rhythm. Curiously, these clinical dysfunctions involve changes in the intracellular calcium signaling cascades (Tostes and Wilde, 1997; Lompré, 1999; Wang and Goldhaber, 2004; Schotten et al., 2011). Calcium is ubiquitous in the regulation of cardiac excitation–contraction coupling. Furthermore, a vast number of other cellular functions have been attributed to this important second messenger. The initiation of cardiac excitation–contraction coupling occurs when an action potential is generated and propagated. This event depolarizes the membrane and activates voltage dependent L-type calcium channels (LCCs). As a consequence, extracellular calcium enters the cells following its electrochemical gradient, an event that stimulates the release of calcium stored in the sarcoplasmic reticulum (SR) through a process known as "calcium-induced calcium release" (CICR). Calcium entry via LCCs and calcium release from the SR together lead to a subsequent rise of the sarcoplasmic calcium concentration ($[Ca^{2+}]i$) to values close to 1 μM, allowing calcium binding to troponin C. The latter event activates the contractile machinery to produce contraction. For the purpose of promoting relaxation, $[Ca^{2+}]i$ must return to its resting levels (~100–150 nM). In this process, two transporters are relevant: SERCA – pumping Ca^{2+} back to the SR – and the Na–Ca^{2+} exchanger (NCX) – extruding Ca^{2+} into the extracellular medium (Bers, 2002, 2008). It is important to mention that insufficient calcium influx leads to an inefficient cardiac contraction. On the other hand, excessive calcium entry should evoke cell death, contracture and generation of pathological membrane currents (Wang and Goldhaber, 2004). For these reasons, the cardiomyocytes have several control points to maintain calcium homeostasis. Among them, the SERCA and LCC activity are significant because in adult cardiomyocytes LCC is the primary route for calcium entry into the cell and SERCA is the most relevant mechanism that reduces $[Ca^{2+}]i$ by promoting calcium reuptake into the SR lumen (Bers, 2002). Our data provide indirect and direct evidence that the AqF of *C. spiralis* reduces calcium influx by blocking LCCs. Because the SR calcium load available for CICR depends on the amount of calcium that enters the cell through activated LCCs, the inhibitory action of AqF would result in a reduction of the global intracellular calcium transient, as we observed in this study. Furthermore, these observations at the functional and cellular levels could account for the cardiodepressor effect of *C. spiralis* in atrial muscle.

Abnormal calcium signaling is involved in the electrogenesis of cardiac arrhythmias. Calcium-mediated arrhythmias can be observed during digitalis toxicity that occurs under conditions of intracellular calcium overload (Clusin, 2003). In this situation, the intracellular sodium concentration rises and the sodium is exchanged for extracellular calcium. The $[Ca^{2+}]i$ overload results in spontaneous SR calcium release by ryanodine receptor (RyR) activation, thereby generating a depolarizing inward current through NCX. This event causes delayed after depolarizations (DADs) and is responsible for the triggered arrhythmias commonly found in heart failure. Another source of triggered arrhythmias is early after depolarizations (EADs). In this case, arrhythmia is caused by prolongation of the action potential, an event that results in an excessive inward calcium current during each cardiac cycle (Wang and Goldhaber, 2004; Schotten et al., 2011). Thus, the mechanisms involved in intracellular calcium handling represent an interesting target for antiarrhythmic drugs. Here we demonstrated that *C. spiralis* is able to modify calcium handling through a reduction in sarcolemmal calcium inward current, which at least in theory, would render the cardiomyocytes less susceptible to arrhythmias. Thus, our present findings support the popular use of teas and infusions obtained from this plant as antiarrhythmic agents. Moreover, we showed that *C. spiralis* blocks L-type calcium channels that are an important molecular target in conventional pharmacotherapy of hypertension, a pathology for which *C. spiralis* is also traditionally used. Although the empirical use of *C. spiralis* does, in fact, have a rationale, it is quite important to highlight the need for further chemical studies and *in vivo* toxicological/pharmacological evaluations of the plant extract to determine its therapeutic efficacy and safety.

5. Conclusions

C. spiralis leaf fractions depress myocardial contractility. The cardioactive compound must be of high polarity because the aqueous fraction, obtained from the crude hydroethanolic extract, was the most potent in reducing atrial contractile force. Its effect does not involve any potassium-linked cellular mechanisms. However, the AqF reduced the L-type calcium current. Despite the lack of quantitative data in the literature about the therapeutic dose of *C. spiralis* when used in folk medicine, our findings support the use of this plant when prescribed for the treatment of arterial hypertension or disturbances of the cardiac rhythm.

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